Multiple regulatory genes in the tylosin biosynthetic cluster of *Streptomyces fradiae*

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Background: The macrolide antibiotic tylosin is composed of a polyketide lactone substituted with three deoxyhexose sugars. In order to produce tylosin efficiently, *Streptomyces fradiae* presumably requires control mechanisms that balance the yields of the constituent metabolic pathways together with switches that allow for temporal regulation of antibiotic production. In addition to possible metabolic feedback and/or other signalling devices, such control probably involves interplay between specific regulatory proteins. Prior to the present work, however, no candidate regulatory gene(s) had been identified in *S. fradiae*.

Results: DNA sequencing has shown that the tylosin biosynthetic gene cluster, within which four open reading frames utilise the rare TTA codon, contains at least five candidate regulatory genes, one of which (*tylP*) encodes a γ-butyrolactone signal receptor for which *tylQ* is a probable target. Two other genes (*tylS* and *tylT*) encode pathway-specific regulatory proteins of the *Streptomyces* antibiotic regulatory protein (SARP) family and a fifth, *tylR*, has been shown by mutational analysis to control various aspects of tylosin production.

Conclusions: The *tyl* genes of *S. fradiae* include the richest collection of regulators yet encountered in a single antibiotic biosynthetic gene cluster. Control of tylosin biosynthesis is now amenable to detailed study, and manipulation of these various regulatory genes is likely to influence yields in tylosin-production fermentations.

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Introduction

Tylosin, a macrolide antibiotic produced by *Streptomyces fradiae*, consists of a polyketide lactone substituted with three deoxyhexose sugars. The structural genes for tylosin biosynthesis (*tyl* genes) are clustered within a defined region (~85 kb) of the *S. fradiae* genome, and are flanked by the resistance determinants *tlrB* and *tlrC* [1,2]. This collection of 43 genes also includes a small number of open reading frames (orfs) that are unassigned and/or might not be essential for tylosin production, but no candidate regulatory gene(s) had been identified in the *tyl* cluster prior to the present work.

Antibiotic biosynthetic gene clusters in actinomycetes typically include pathway-specific regulatory genes that may themselves be controlled in a 'cascade' fashion by additional regulatory elements (for review, see [3]). The latter, which are not usually found in antibiotic biosynthetic clusters, might exert pleiotropic control over multiple pathways of secondary metabolism (as in *Streptomyces coelicolor*, which produces four different antibiotics) or might regulate both antibiotic production and morphological differentiation. However, comparable data have not been reported with macrolide-producing organisms. The much-studied *ery* cluster of *Saccharopolyspora erythraea* contains no regulatory genes [4–7], and none that influences erythromycin production has been found elsewhere within the *S. erythraea* genome. Only two genes have hitherto been shown to regulate aspects of macrolide production. The first of these, *srmR* in the spiramycin producer *Streptomyces ambofaciens*, is required for transcription from the promoters of *srmG* (which encodes a polyketide synthase) and *srmX*, a gene of unknown function [8]. The other, *acyB2* of *Streptomyces thermotolerans*, was shown [9] to activate expression of the adjacent gene, *acyB1* (also known as *carE*; [10]), which encodes 4″-*O*-acyltransferase activity required during carbomycin biosynthesis. In short, prior to the present work almost nothing was known about the transcriptional regulation of macrolide biosynthesis.

Here we present the sequence of two regions of the *S. fradiae tyl* gene cluster within which we have identified at least five candidate regulatory genes, one of which has been subjected to mutational analysis.

Results

Sequence analysis of *tyl* DNA

Two blocks of *S. fradiae tyl* DNA were sequenced in the present work. The first (3085 base pairs, accession number AF145042), located upstream of *tylG*, revealed two orfs, one complete and one incomplete. The latter was the continuation of an incomplete orf located at the end of the *tylIBA*

sequence determined previously (accession number U08223; [11]) and allowed reconstruction of *orf6* (Figure 1), which is co-directional with the six preceding orfs. The complete orf sequenced here (*orf7*) is convergent with *orf6* and the respective TGA stop codons are separated by 139 bp that includes a prominent pair of inverted repeat sequences. The present sequence extends 604 bp upstream of *orf7* and terminates 377 bp before the start of *orf8*.

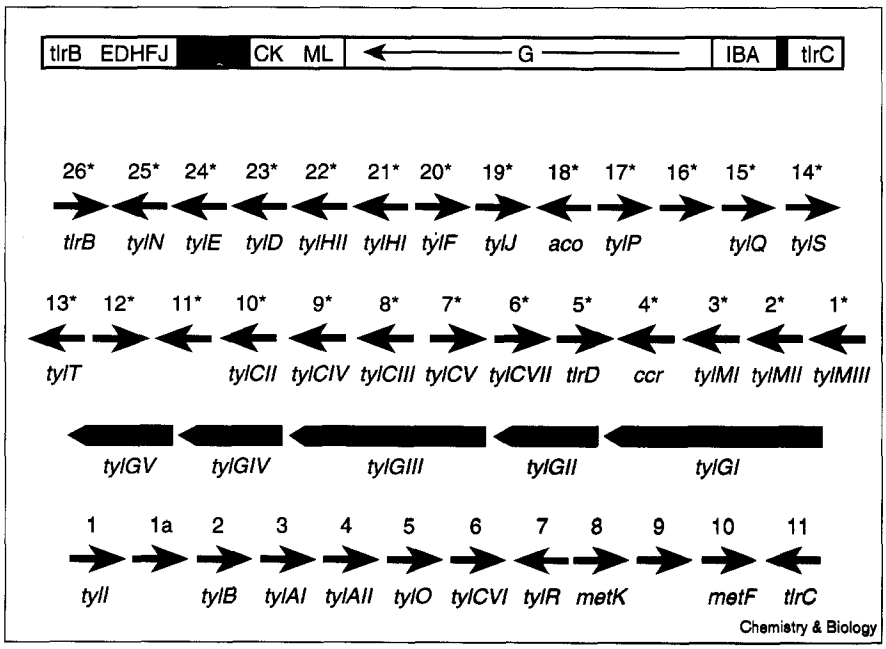
The other block of sequence analysed here (10,467 bp; accession number AF145049) was derived from DNA downstream of *tylG* (Figure 1), between clusters of structural genes that encode the biosynthesis of mycarose (*orf6*–orf10** from the *tylCK* region; N.B., A.R.B, I.P. Smith and E.C., unpublished observations; accession number AF147704) and mycinose (*orf19*–orf25** covering *tylEDHFJ* [12]; Genbank accession number AF147703). The present sequence contains eight complete orfs (*orf11*–orf18**) plus 50 bp at either end. At the left-hand end in the orientation of Figure 1, the sequence terminates within the 160 bp gap that separates *orf18** from the convergent *orf19** (*tylJ*), and overlaps by 209 bp the sequence given under AF147703. At the right-hand end, the present sequence terminates 21 bp inside *orf10** (*tylCII*). The orfs described below are introduced in functional groups and not in numerical order.

Assignment of regulatory orfs

orf7 (tylR): *a global regulator of tylosin production*

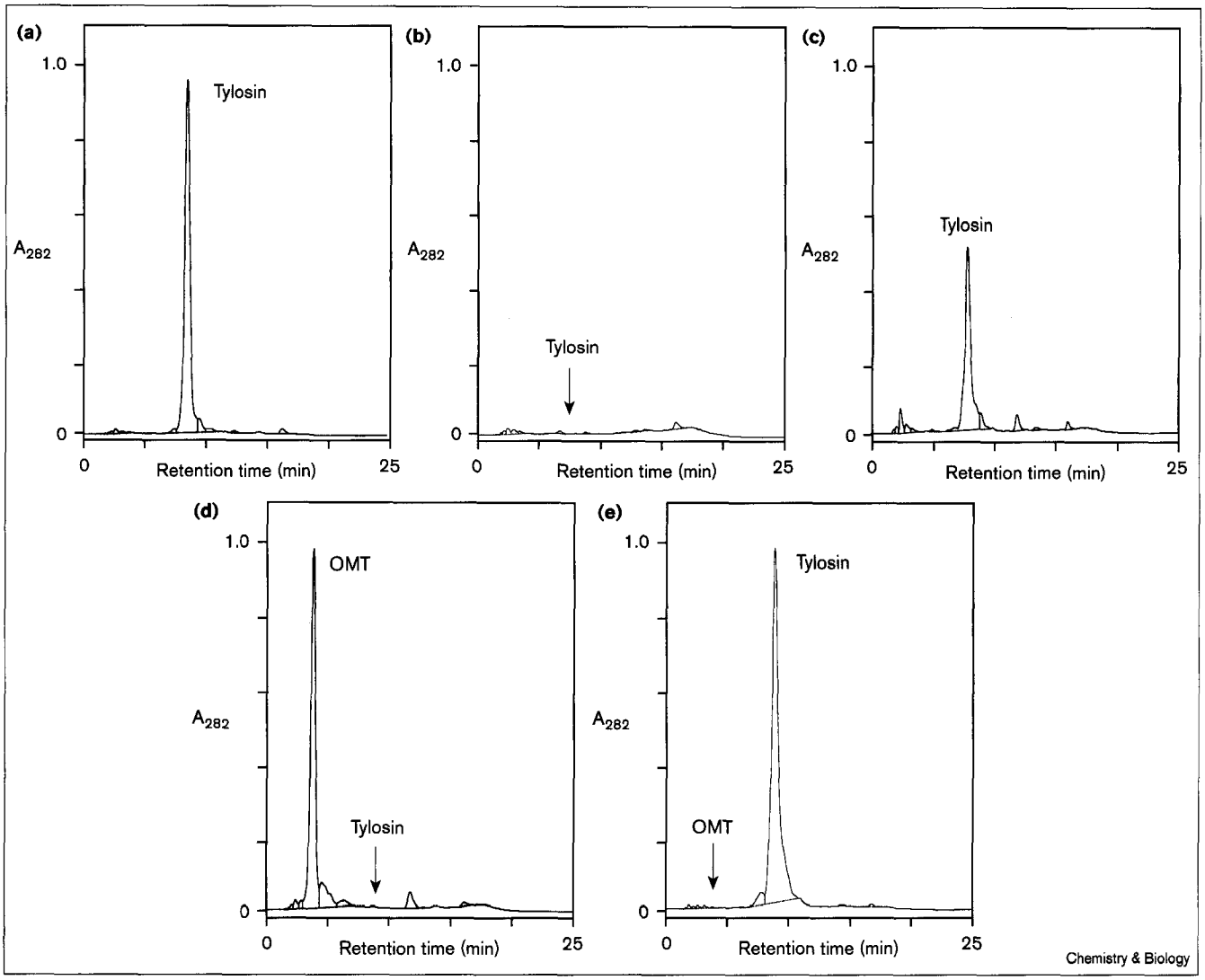
The deduced product of *orf7* (430 amino acids maximum, Mr 46,250) displays end-to-end similarity (and 42% sequence identity) to the product of *acyB2* from *S. thermotolerans*, producer of carbomycin [9]. Given that *acyB2* was one of the first (and few) regulatory genes to be identified among macrolide-producing organisms, the function of *orf7* was addressed using targeted gene disruption, utilising the hygromycin B resistance cassette, Ωhyg [13]. This was done without affecting the expression of downstream genes because *orf6* and *orf7* are convergent. Having confirmed the chromosomal disruption by Southern analysis (data not shown), the *orf7*-disrupted strain was introduced into tylosin-production medium and fermented. However, very little material absorbing at 282 nm was detectable by high-performance liquid chromatography (HPLC) analysis of the fermentation products (Figure 2b). In contrast, when intact *orf7* (together with *ermEp**) was integrated into the ϕC31 *attB* site of the *orf7*-disrupted strain, significant levels of tylosin were produced (Figure 2c) although not as high as those normally seen with the wild type strain (Figure 2a). To ascertain which aspect of tylosin production was affected, fermentations involving the *orf7*-disrupted strain were supplemented with various intermediates of the tylosin biosynthetic pathway. These included the aglycone (tylactone), precursors of tylosin lacking one or more sugars, and also macrocin and demethyl-macrocin that, respectively, lack one or both of the *O*-methyl groups that are added during the last two steps of tylosin production. The results were unequivocal. Each of the added compounds was recovered intact following fermentation, with no detectable bioconversion to later intermediates in the pathway or to

Figure 1



The tylosin-biosynthetic gene cluster of *S. fradiae*. The resistance determinants, *tlrB* and *tlrC*, are about 85 kb apart in the genome and flank 13 loci (*tylA–M*) that were identified by complementation analysis and cross feeding studies using blocked mutants of *S. fradiae* [1,2,45]. The *tylG* locus covers about 41 kb and contains five polyketide synthase genes reading right to left. Upstream of *tylG*, 12 genes (*orf1*, *orf1a*, *orf2–orf11*) including *tlrC* occupy about 15 kb. Downstream of *tylG*, 26 genes (*orf1*–orf26**) including *tlrB* occupy about 29 kb. Complete orfs sequenced here are shown in red. All of the structural genes required for tylosin production appear to lie between *tlrB* and *tlrC*, but it remains to be established whether tylosin production is influenced by additional genes outside the cluster, as presently defined.

Figure 2



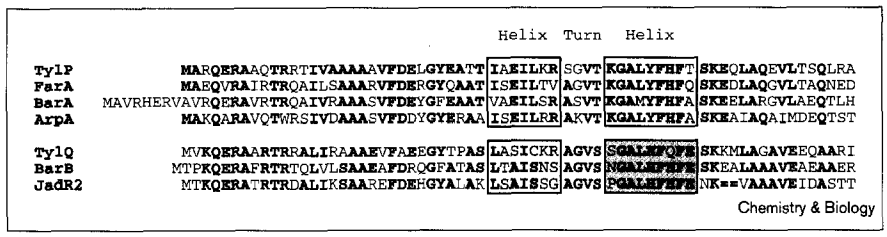
Fermentation products from strains of *S. fradiae*. HPLC analysis of material produced by: **(a)** wild type; **(b)** an *orf7*-disrupted strain; **(c)** an *orf7*-disrupted strain complemented with *orf7*; **(d)** an *orf7*-disrupted strain fed *O*-mycaminosyl-tylonolide (OMT); **(e)** wild type supplemented with OMT. Tylonolide (20,23-*bis*-hydroxy-tylactone), is not an intermediate in the tylosin pathway but could formally be produced from tylosin if all three sugars were removed hydrolytically.

tylosin itself (for data obtained using the tylosin precursor, *O*-mycaminosyl-tylonolide (OMT), see Figure 2d). In controls, the same compounds were added to fermentation cultures of the *S. fradiae* wild type strain and each was quantitatively converted to tylosin (the bioconversion of OMT is illustrated in Figure 2e). Evidently, disruption of *orf7* shuts down most, if not all, aspects of tylosin biosynthesis, including polyketide metabolism, synthesis or addition of all three sugars, as well as terminal *bis O*-methylation. Such consequences would typically result from disruption of a positive regulatory element that might normally control multiple tylosin biosynthetic promoters and/or might activate other hierarchical regulator(s). This conclusion is consistent with the earlier suggestion that *acyB2* encodes a positive regulator [9]. Given that *orf7* was the first regulatory gene encountered in the *tyl* cluster it was designated '*tylR*' although, as detailed below, several additional candidates have since been identified.

orf17* (tylP) encodes a γ-butyrolactone receptor

The deduced *orf17** product shows convincing end-to-end matches, with greatest conservation in the amino-terminal regions (Figure 3), to various well-characterised γ-butyrolactone receptor proteins, including FarA (the IM-2 receptor from *Streptomyces sp.* [14]), ArpA (the A-factor binding

Figure 3



Amino-terminal sequences of TylP, TylQ and similar proteins in the database. Comparison with the experimentally determined amino-terminal sequences of FarA, ArpA and BarA [14–16] allowed the likely starts of BarB [18] and TylP (present work, see text) to be deduced. The TylQ sequence shown corresponds to the longest possible product of *orf15**. The start of the JadR2 sequence was also inferred, in part, from the G+C content of the upstream DNA [20]. These proteins range in length from 196 ($JadR_2$) to 276 (ArpA) amino acids but only the highly conserved amino-terminal sequences are shown. The helix–turn–helix motifs are indicated; the downstream 'recognition' helix that binds into the major groove of the DNA target site is especially well conserved and differs between the two groups of proteins. Accession numbers: *tylP*, AF145049; *barA*, D32251; *farA*, AB001683; *arpA*, D49782; *tylQ*, AF145049; *barB*, AB001609; *jadR*$_2$, U24659.

protein from streptomycin-producing *Streptomyces griseus* [15]) and BarA (the butanolide receptor from *Streptomyces virginiae*; producer of virginiamycin [16]). By comparison with the known sequences of these three proteins, the product of *orf17** is probably 226 amino acids long (Mr 24,800) although the orf could, theoretically, encode a product 90 residues longer. CODONPREFERENCE analysis is also compatible with this interpretation. The γ-butyrolactones, a family of closely related (but strain-specific) low molecular weight signalling factors, are often alluded to as *Streptomyces* hormones and act pleiotropically to switch on morphological differentiation and secondary metabolism. For example, in *S. griseus*, A-factor controls streptomycin production and resistance, and also regulates aerial mycelium formation [17]. The receptors for these signalling molecules are typically repressors. Thus in *S. virginiae*, BarA binds to the promoter of a downstream gene, *barB* [18,19], and induction of virginiamycin production requires γ-butyrolactones, the so-called virginiae butanolides (VBs). Consistent with their DNA-binding functions, the various butyrolactone-binding proteins possess amino-terminal helix–turn–helix motifs that are highly conserved and the *orf17** protein (TylP) clearly resembles BarA and others in this respect (Figure 3). We conclude that TylP is a butyrolactone-responsive regulator of undetermined function, although precedent suggests that it might be a transcriptional repressor.

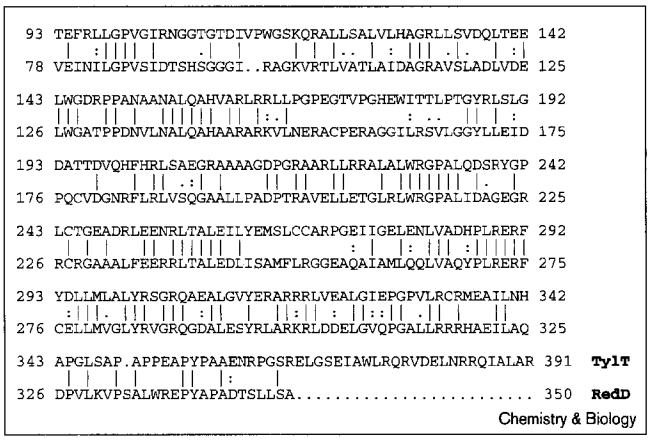
orf15* (tylQ): a candidate target for TylP

By far the closest sequence matches to the deduced *orf15** product (213 amino acids, Mr 23,100) were to $JadR_2$ from *Streptomyces venezuelae*, the jadomycin B producer [20], and BarB from the producer of virginiamycin [18]. In the genome of *S. virginiae*, *barB* lies immediately downstream of *barA* and is negatively controlled by the *barA* product. In the presence of VBs, BarA dissociates from the *barB* promoter and transcription of *barB* is ostensibly facilitate DNA-binding (Figure 3), suggesting that the product of *barB* might be a second transcriptional regulator that functions downstream of BarA and VBs in the regulatory cascade that controls virginiamycin production [18]. By analogy, a similar model might link the products of *orf17** and *orf15**, which are also related to each other (34% sequence identity) and are, respectively, similar to BarA and BarB. Comparison of the helix–turn–helix motifs, particularly the downstream 'recognition' helices that bind to DNA, is also compatible with division of these various proteins into two groups (Figure 3). Although no detailed function has been suggested for BarB, analogies between these various systems raise the possibility that it might be a transcriptional activator. In this scenario, the A-factor receptor of *S. griseus* (ArpA) represses 'gene X', which encodes a transcriptional activator involved in the regulatory pathways for both streptomycin production and aerial mycelium formation (for review see [17]). By analogy, it is also possible that *tylQ* is a transcriptional regulator controlled by TylP.

orf13* (tylT) and orf14* (tylS) encode pathway-specific regulatory proteins

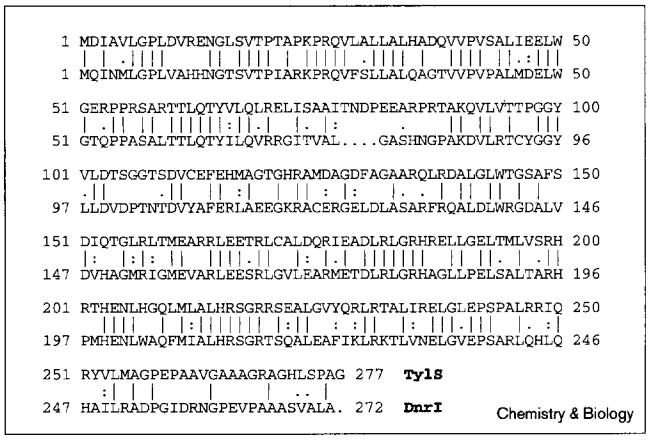
The deduced products of *orf13** and *orf14** are distinctly similar to each other (with 42% sequence identity) and both obviously belong to the growing family of pathway-specific activators known as SARPs (*Streptomyces* antibiotic regulatory proteins; [21]). Although its match to the Orf14* sequence is closer than to any in the database, Orf13* nevertheless displays striking similarity to the amino-terminal region of AfsR of *S. coelicolor* [22] and to RedD (42% identity; Figure 4), the pathway-specific activator of the undecylprodigiosin biosynthetic genes of the same organism [23]. Similarly, the *orf14** product displays convincing end-to-end matches to these and other SARPs, especially DnrI (50% identity; Figure 5) from *Streptomyces peucetius*, the daunorubicin producer [24], and the products of *gra orf9*

Figure 4



GAP comparison of the deduced sequences of TylT and RedD. The amino-terminal sequences of these proteins have not been determined experimentally. They have been inferred by comparison of DNA sequences encoding these and other SARPs (notably ActII-orf4, see text) and do not correspond to the longest possible products of the respective orfs. The accession number of RedD is AL021530.

Figure 5



GAP comparison of the deduced sequences of TylS and DnrI. The amino-terminal sequences of these proteins have not been determined experimentally. They were inferred by comparison of DNA sequences encoding these and other SARPs (see text) and do not correspond to the longest possible products of the respective orfs. The accession number of DnrI is M80237.

a less close match (38% sequence identity) to ActII-orf4, the pathway-specific activator of the *S. coelicolor* actinorhodin cluster [25], although that is closer than the match between ActII-orf4 and RedD. The amino-terminal sequences of these various SARPs have not been determined experimentally. They were deduced from the DNA sequences that encode them and translational starts in the respective orfs were assigned by matching the positions of alternative candidate start codons. As a result, the deduced proteins do not necessarily correspond to the longest possible products of the various orfs. For example, Orf14* (TylS) is probably 277 amino acids long (Mr 30,100), although the gene could theoretically encode a protein of 293 residues. Similar considerations (compatible with CODONPREFERENCE analysis), suggest that the *orf13** product (TylT) might also be shorter than the maximum possible size of 404 amino acid residues. Similar to other SARP-encoding genes, *orf13** and *orf14** both contain a TTA codon, encoding Leu324 and Leu69, respectively. Actinomycetes have extremely GC-rich DNA and rarely use TTA codons, which are typically encountered only in resistance determinants or regulatory genes of secondary metabolism [26].

orf11*, *an additional regulatory orf?*

The deduced *orf11** product (425 amino acids maximum; Mr 45,400) is extremely similar over its whole length to a hypothetical ATP/GTP-binding protein encoded by *SC4H2.17* of *S. coelicolor* (accession number AL022268) and also shows an end-to-end match to HflX of *Escherichia coli*, a component of the HflA complex of three proteins that also includes HflK and HflC. Both HflB (synonym FtsH) and HflA were described as proteases that cleave protein cII of bacteriophage lambda, thereby reducing the frequency of lysogenisation [27,28] and HflX (a putative GTPase protein) was proposed to regulate such activity of HflKC [29]. More recently [30], it was suggested that FtsH, an ATP-dependent zinc metalloprotease, is the protease that degrades protein cII and that membrane-associated HflKC inhibits such activity. This latter model contained no precise role for HflX, but we are intrigued to learn (A. Wietzorrek, personal communication) that the gene immediately adjacent to *SC4H2.17* in the *S. coelicolor* chromosome is deduced to encode a zinc metalloprotease. We suspect that the *orf11** product might somehow be involved in regulated proteolysis. Other GTP-binding (Obg) proteins that are distantly related to HflX are postulated to regulate morphological differentiation in *S. griseus* and *S. coelicolor* [31,32] but there is currently no evidence linking Orf11*, or its orthologue in *S. coelicolor*, to sporulation.

Assignment of other TTA-containing orfs

Hitherto, the observed usage of TTA codons by actinomycetes was confined to genes involved in resistance or regulation of secondary metabolism. Although plausible roles in the regulation of tylosin production can be posited for TylS and TylT, the presence of TTA codons encoding Leu26 and Leu59 in *orf18** and *orf16**, respectively, is less readily rationalised.

orf18* *encodes acyl-CoA oxidase*

The deduced product of *orf18** (641 amino acids maximum, Mr 69,900) is similar over much of its length to various acyl-CoA oxidases, authentic and hypothetical. The closest match was to the product of *aco* from *Myxococcus xanthus* (accession number AF013216) but convincing similarities were also seen to deduced proteins from

Arabidopsis thaliana (AF057043) and *Cucurbita* sp. (AF002016), and to an authentic peroxisomal pristanoyl-CoA oxidase from rat [33]. Given that acyl-CoA oxidases initiate β-oxidation of fatty acids, Orf18* might help to provide short-chain acyl CoA substrates for polyketide metabolism and/or the synthesis of γ-butyrolactone(s).

orf16* encodes a cytochrome P450

The deduced product of *orf16** contains, at most, 433 amino acids (Mr 47,000), although alternative candidate start codons could give rise to a shorter product. Orf16* is evidently a cytochrome P450 and gives end-to-end matches to many such sequences in the database, particularly the product of *mycG* from the mycinamicin producer, *Micromonospora griseorubida* [34]. The *orf16** product displays highly conserved sequence motifs [35] characteristic of cytochromes P450, including the binding pocket containing the invariant cysteine involved in haem attachment (**FG**HG**V**HY**C**LG**A**P**L**AR**L**E**A**GI, using single-letter amino acid code; consensus sequence given in bold). Further upstream, there is a clearly recognisable oxygen-binding motif (AGAES, a variant on the consensus sequence AGxET that is also seen, as AGYES, in the *mycG* product). During analysis of a *M. griseorubida* mutant blocked in mycinamicin II production, bioconversion and complementation analysis suggested that the product of *mycG* was remarkable in possessing two separate activities, namely, 12,13-epoxidation and 14-hydroxylation on the polyketide ring [34]. Interestingly, PikC of *S. venezuelae* (which is closely similar to MycG and to the *orf16** product) also catalyses multiple hydroxylations, at C-12 in the conversion of narbomycin to pikromycin, and at C-10 and C-12 in the conversion of YC-17 to methymycin and neomethymycin, respectively [36]. Because the ring hydroxylations (at C-20 and C-23) required during tylosin production are catalysed by the products of *tylI* and *tylHI*, respectively ([11,12,37]), the role of the *orf16** product remains elusive.

orf12* is unassigned

The deduced product of *orf12** is a protein of 212 amino acids maximum (Mr 22,500), the sequence of which is unlike any in the database. *orf12** is one of only three unassigned orfs in the *tyl* cluster. The other two (*orf1a* and *orf9*) are located upstream of *tylG*, over 50 kb away from *orf12**. As discussed above, the start of the TylT coding sequence is not known with certainty, and the gene might not fill the whole of *orf13**. If not, there could be room for an additional short orf (upstream of, and divergent from, *orf12**) encoding a deduced product of 68 amino acids that finds no match in the database. The significance (if any) of this sequence remains to be established.

Discussion

Compared with other antibiotic biosynthetic gene clusters, the *tyl* cluster displays unprecedented features, including a multiplicity of regulatory genes (two of which encode SARPs) with four orfs that utilise the rare codon TTA. The presence of signal transduction genes is also remarkable. Although γ-butyrolactone signalling factors are widespread (and probably ubiquitous) among the actinomycetes (for review, see [38]), genes that encode their receptors and transmit the signals are not commonly found among those that encode antibiotic biosynthesis.

The regulatory genes of the *tyl* cluster are all preceded by noncoding 'gaps' that range in size from 128 bp upstream of *tylP* to 981 bp upstream of *tylR*. Moreover, because the *tylP*, *tylS* and *tylT* coding sequences might be shorter than their theoretical maximum lengths, it is likely that each of the five regulators is preceded by an upstream gap of greater than 300 bp. These noncoding regions presumably allow independent expression of the respective genes.

As a working hypothesis, purely on the basis of precedent, TylP is proposed to be a butyrolactone-responsive transcriptional regulator, perhaps a repressor. A likely, but not necessarily unique, target for TylP is *tylQ*, the product of which might regulate structural genes of the tylosin cluster and/or one or both of the pathway-specific regulatory genes, *tylS* and *tylT*. Precise roles for the latter two genes remain to be defined. TylR influences polyketide and deoxyhexose metabolism but does not appear to affect morphological differentiation. The hierarchical order of involvement of these (and perhaps other) genes in the regulatory cascade that governs tylosin production remains to be established.

Significance

The tylosin biosynthetic (*tyl*) gene cluster of *Streptomyces fradiae* is only the second example of a completely sequenced set of structural genes for the production of a macrolide antibiotic, the other being the much studied erythromycin biosynthetic (*ery*) gene cluster of *Saccharopolyspora erythraea*. What makes the *tyl* cluster particularly interesting is the presence of so many regulatory genes. Other antibiotic biosynthetic gene clusters are not known to contain multiple pathway-specific regulators, and the presence in the *tyl* cluster of genes associated with signal transduction, involving diffusible microbial hormones, is also unprecedented. In contrast, no regulatory genes are present in the *ery* cluster, and none that affects erythromycin production has yet been found elsewhere in the *S. erythraea* genome. The regulatory genes identified here probably control tylosin biosynthesis in cascade fashion and might form a link to the control of sporulation. Manipulation of these regulatory genes is expected to influence yields in tylosin production fermentations.

Materials and methods

Bacterial strains, growth conditions and genetic manipulation

S. fradiae T59235 (also known as C373.1, and referred to here as wild type) was maintained and propagated at 37°C on AS-1 agar [39] or at 30°C in tryptic soy broth (Difco). Plasmids were manipulated in *E. coli* using standard protocols [40]. DNA was introduced into *S. fradiae* via conjugal transfer from *E. coli* as described elsewhere [41] using pOJ260 [42] and pLST9828 [43]. pOJ260 is a suicide vector, unable to replicate in *Streptomyces* spp., and was used for targeted gene disruption. pLST9828, used for complementation analysis, integrates into the chromosomal ΦC31 *attB* site and contains a powerful constitutive promoter, *ermEp**, to ensure expression of cloned genes.

Targeted gene disruption via gene transplacement

A 2.1 kb *Sst*I–*Bam*HI fragment containing *orf7* together with flanking DNA was excised from pSET552 [2] and inserted into pIJ2925 [44]. Disruption of the *orf7* coding region involved the unique *Nco*I site, approximately central within the subcloned DNA, into which the hygromycin B resistance cassette, Ωhyg [13] was inserted using blunt-end ligation. This placed Ωhyg, which has flanking transcriptional terminators, 378 bp downstream from the start of *orf7* and 914 bp upstream from the translational stop. The disrupted *orf7* was then ligated, as a *Bgl*II fragment, into the *Bam*HI site of pOJ260 and introduced into *S. fradiae*. Following initial selection on hygromycin B (75 μg ml^{-1}), transconjugants were screened for sensitivity to apramycin (25 μg ml^{-1}) to identify double recombinants in which chromosomal *orf7* had been replaced with the disrupted gene.

Complementation of disrupted strains

A 1.69 kb *Sst*I–*Nru*I fragment from pSET552, containing *orf7* flanked by noncoding DNA (188 bp upstream, 210 bp downstream), was ligated into pLST9828 and thereby introduced into the *orf7*-disrupted strain of *S. fradiae*.

Fermentation analysis

Fermentation of *S. fradiae*, bioconversion of exogenous tylosin precursors and HPLC analysis of products, with internal standards, are described elsewhere [43]. Gene transplacement is a stable event and this, together with the use of integrative plasmids for complementation, eliminated the need for antibiotic selection during fermentation.

DNA manipulation and sequencing

The *S. fradiae tyl* DNA sequenced here was obtained from pHJL311 [45] and from pSET552 [2]. Fragments were subcloned in pIJ2925 [44] and both strands of the DNA were sequenced independently in overlapping fashion by a combination of nested deletion analysis and primer walking. This was carried out on an automated DNA sequencer using fluorescent-dye-labelled dideoxynucleotide chain terminators and *Taq* or *Taq* FS polymerase. DNA sequences together with the corresponding chromatograms were imported into Seq Ed v 1.0.3 and aligned using AUTO ASSEMBLER. Sequences were analysed using the University of Wisconsin GCG software programmes. Open reading frames were identified using CODONPREFERENCE, BLASTX and six-frame translation with DNA STRIDER. Deduced products were analysed using BLASTP.

Accession numbers

The sequences presented in this paper have been deposited in Genbank, and are available under accession numbers AF145042 and AF145049.

Note added in proof

A sequence (accession number AF055922), significantly different from that presented here, has recently been proposed for *orf17** and *orf18** [46].

Acknowledgements

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